Serum-activated assembly and membrane translocation of an endogenous Rac1:effector complex

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Rho family GTPases (Cdc42, Rac1, and RhoA) function downstream of Ras [1], and in a variety of cellular processes [2]. Studies to examine these functions have not directly linked endogenous protein interactions with specific in vivo functions of Rho GTPases. Here, we show that endogenous Rac1 and two known binding partners, Rho GDP dissociation inhibitor (RhoGDI) and p21-activated kinase (PAK), fractionate as distinct cytosolic complexes. A Rac1:PAK complex is translocated from the cytosol to ruffling membranes upon cell activation by serum. Overexpression of dominant-negative (T17N) Rac1 does not affect the assembly or distribution of this Rac1:PAK complex. This is the first direct evidence of how a specific function of Rac1 is selected by the assembly and membrane translocation of a distinct Rac1:effector complex.

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Results and discussion

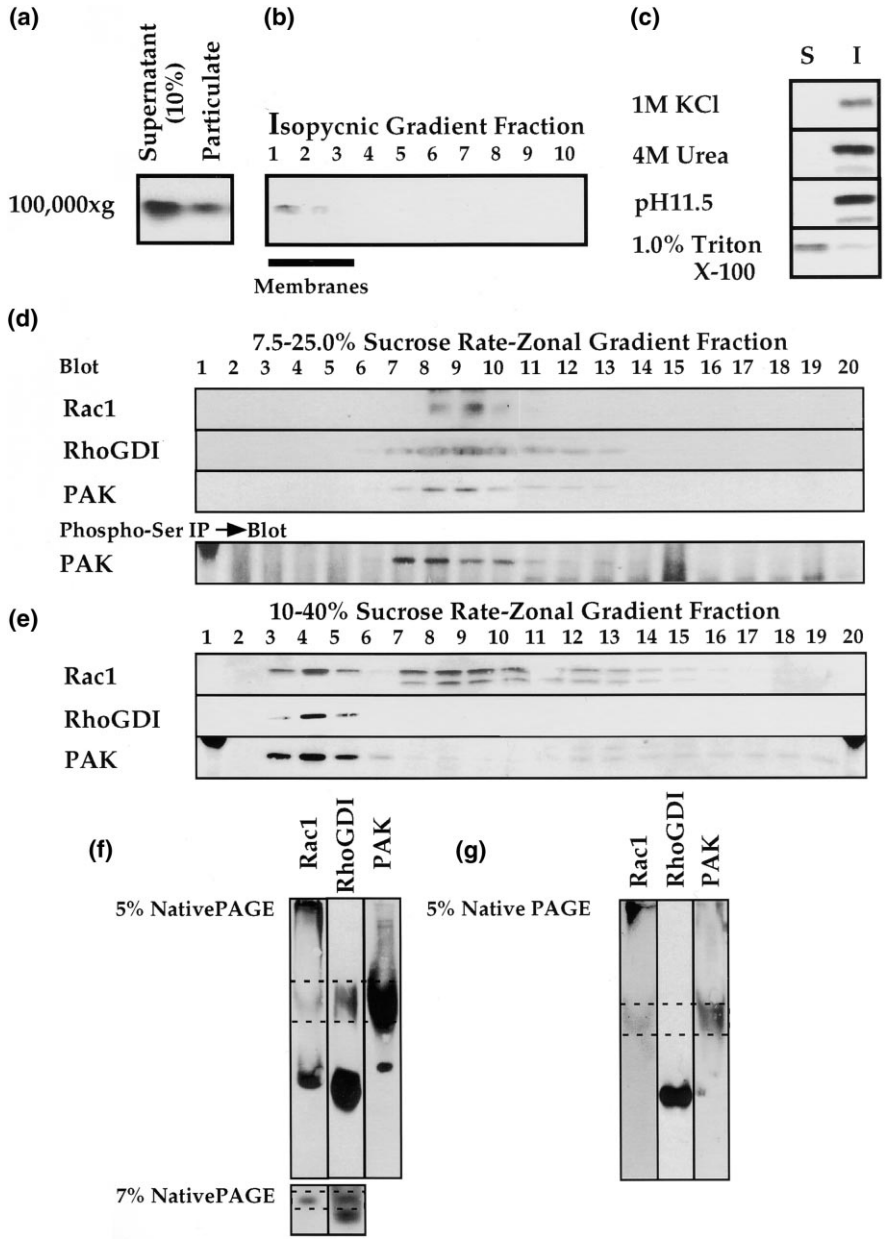
Current models propose that active, GTP-bound Rac1 interacts with downstream effectors on membranes [2] to regulate actin dynamics, gene transcription, membrane trafficking, cell adhesion, cell motility, and transformation [3]. These functions have been examined indirectly by in vitro screening for binding partners [4], and inferred from the effects of overexpressing exogenous dominant-negative and constitutively active GTPase mutants [3]. How endogenous Rac1:effector complexes are assembled and targeted to specific cellular sites is poorly understood.

To examine endogenous Rac1:effector complexes, we fractionated MDCK cell homogenates into cytosol and membranes, and further separated proteins in these fractions by sucrose gradient centrifugation and native polyacrylamide gel electrophoresis. Differential centrifugation of MDCK cell homogenates revealed that >95% of endogenous Rac1 was cytosolic (Figure 1a). Rac1 detected in the particulate fraction floated with membranes upon isopycnic density centrifugation in iodixanol (Figure 1b; note that E-cadherin, a transmembrane protein, floats in these same fractions as Rac1) [5], and was solubilized by nonionic detergent, but not by buffers that remove peripheral membrane proteins (Figure 1c).

To evaluate molecular associations of endogenous Rac1 in the cytosol, cytosolic proteins were separated by rate zonal centrifugation (Figure 1d). Rac1 (21 kDa) sedimented as a single peak with an apparent molecular weight of ~60 kDa. RhoGDI (29 kDa), which binds Rac1 in vitro to inhibit the release of bound GDP [6, 7] and disengages Rac1 from membranes [8], and PAK (62 kDa), another Rac1 binding partner [9] that has been implicated in initiating kinase cascades [10], actin dynamics [11, 12], and Ras-mediated transformation [13], cosedimented with Rac1. Activated PAK autophosphorylates on serine and threonine residues [9]. We tested for the presence of active phospho-PAK in cytosol by immunoprecipitating sucrose gradient fractions with phosphoserine antibodies and Western blotting with a PAK antibody. Phosphoserine antibodies immunoprecipitated PAK in a peak that did not coincide with the majority of PAK or with Rac1 and RhoGDI (Figure 1d, lowest panel), indicating that the population of PAK cofractionating with Rac1 is not active. Cytosolic proteins were further resolved by native PAGE [14]. Rac1 migrated as two distinct bands, and RhoGDI comigrated with both of those bands (Figure 1f) as well as with a third, faster migrating band that was resolved in 7% native PAGE and did not contain Rac1 (Figure 1f). PAK was detected in two bands: a band with reduced electrophoretic mobility that comigrated with the slowest migrating bands of Rac1 and RhoGDI, and a fast migrating band (Figure 1f).

To identify Rac1 complexes on membranes, extracts from MDCK cell membranes were separated by rate zonal centrifugation (Figure 1e). Rac1, RhoGDI, and PAK cosedimented in a peak with an apparent molecular weight of ~60 kDa. Rac1 also appeared in other faster sedimenting fractions; these larger protein complexes will be described elsewhere (M.D.H.H. and W.J.N., unpublished data). Extracts from MDCK cell membranes were further resolved by native PAGE. Rac1 and PAK comigrated as a single band (Figure 1g) with an electrophoretic mobility greater than that of the cytosolic Rac1, RhoGDI, and PAK (Figure 1f). RhoGDI resolved into a single band (Figure 1g) with an electrophoretic mobility similar to that of the fast mi-

Figure 1



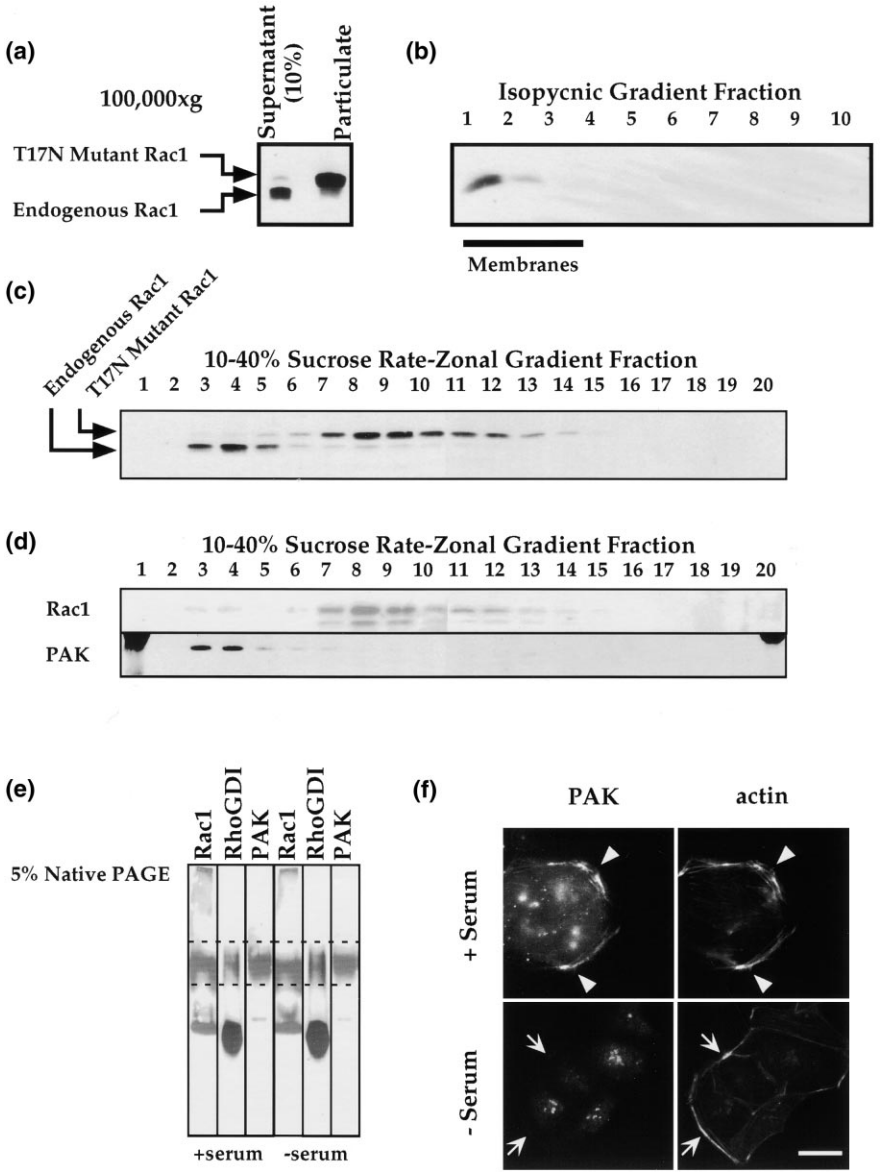
Identification of endogenous Rac1 protein complexes. **(a)** Distribution of Rac1 in particulate and supernatant fractions following differential centrifugation of a postnuclear supernatant from MDCK cells. **(b)** Rac1 distribution in iodixanol gradients following isopycnic centrifugation [5] of the particulate fraction. **(c)** Solubility (S, soluble; I, insoluble) properties of Rac1 following the incubation of membranes (see [a]) with 1 M KCl, 4 M urea, bicarbonate buffer (pH 11.5), or 1% (v/v) Triton X-100 in buffer A. **(d)** Sedimentation profile of cytosolic Rac1, RhoGDI, and PAK in 7.5%–25.0% (w/v) sucrose gradient following rate zonal centrifugation of MDCK cell cytosol. Note that the PAK immunoblot in the lowest panel is of phosphoserine immunoprecipitations from gradient fractions. **(e)** Sedimentation of membrane-extracted Rac1, RhoGDI, and PAK in a 10%–40% sucrose gradient following rate zonal centrifugation. **(f)** Native 5% and 7% polyacrylamide gel electrophoresis of cytosolic proteins. Replicate lanes of protein samples were separated in the same gel, transferred to a PVDF membrane, cut out, and then separately immunoblotted with each antibody before being reassembled for exposure to X-ray film. Boxed areas denote comigration of multiple proteins. **(g)** Native 5% polyacrylamide gel electrophoresis of extracted proteins from the particulate fraction shown in Figure 1a. Boxed areas denote comigration of multiple proteins.

grating band containing only RhoGDI from the cytosol (Figure 1f).

Dominant-negative (T17N) Rac1 is thought to act by sequestering proteins required for the activation of endogenous Rac1 [15, 16]. MDCK cells expressing T17N Rac1 under the control of the Tet repressor [17] were used to directly test this hypothesis. Unlike endogenous Rac1 (Figure 1a), >95% of T17N Rac1 fractionated with membranes upon either differential (Figure 2a) or isopycnic centrifugation (Figure 2b). Extracted membranes from T17N Rac1–expressing cells were subjected to rate zonal centrifugation. In the presence of T17N Rac1, endogenous Rac1 sedimented as a ~60 kDa complex, exactly as in control cells. T17N Rac1 sedimented with an apparent molecular weight of ~225,000 Da (Figure 2c).

Serum activation initiates Ras signaling, inducing a burst of actin polymerization and membrane ruffling that requires Rac1 [1]. PAK has been shown to localize to ruffling membranes in serum-treated fibroblasts [18]. We asked how serum affects endogenous Rac1 and PAK. Cytosol from MDCK cells grown in the presence or absence of serum was subjected to native PAGE. An identical pattern of Rac1, RhoGDI, and PAK bands were detected under both conditions (Figure 2e). Membrane extracts from

Figure 2



Regulation of Rac1:effector complex formation. **(a)** Distribution of endogenous Rac1 and T17N Rac1 in particulate (P) and supernatant (S) by differential centrifugation of MDCK cell postnuclear supernatant. The Rac1 antibody recognizes both endogenous and T17N Rac1, and the latter migrates slower than endogenous Rac1 because of the addition of a myc epitope amino acid sequence [17]. **(b)** T17N Rac1 distribution in an iodixanol gradient following isopycnic centrifugation [5] of the postnuclear supernatant from T17N Rac1–expressing MDCK cells. 9E10 myc antibody was used for Western blotting to detect T17N Rac1. **(c)** Sedimentation profile of endogenous Rac1 and T17N mutant Rac1 from an extracted particulate fraction fractionated on a 10%–40% sucrose gradient by rate zonal centrifugation. **(d)** Sedimentation of membrane-extracted Rac1 and PAK from MDCK cells grown in the absence of serum following rate zonal centrifugation in a 10%–40% sucrose gradient. Compare to Figure 1e. **(e)** Native 5% polyacrylamide gel electrophoresis of cytosolic proteins from MDCK cells grown in the presence or absence of serum (see Figure 1f, legend). Boxed areas denote comigration of multiple proteins. **(f)** Distributions of actin and PAK in MDCK cells that were cultured on collagen-coated glass coverslips in the absence or presence of serum. Actin was stained with rhodamine-phalloidin and PAK was detected with a PAK antibody and a FITC-labeled secondary antibody. Note the localization of PAK at ruffling membranes in cells that were grown in the presence of serum (arrowheads), but the lack of ruffling membranes and membrane staining of PAK in cells grown in the absence of serum (arrows). The scale bar represents 20 μm.

MDCK cells grown without serum were separated by rate zonal centrifugation (Figure 2d). In the absence of serum, Rac1 did not sediment as a 60-kDa peak, while PAK still sedimented in those fractions.

The distribution of endogenous PAK was determined by immunofluorescence. PAK localized to membrane ruffles and internal tubulovesicular structures. The staining of PAK at ruffling membranes colocalized with cortical actin. When cells were cultured in the absence of serum, however, PAK was not localized to ruffling membranes, although tubulovesicular staining remained (Figure 2f).

To our knowledge, this is the first study to examine the organization of endogenous Rac1 in cells in response to a specific extracellular signal. We separated proteins by sucrose gradient centrifugation and native PAGE, which provide quantitative and nonselective surveys of protein complexes, and found that RhoGDI and PAK cofractionated with Rac1. The cofractionation of these proteins under different conditions indicates the formation of specific protein complexes comprising Rac1, RhoGDI, and PAK in the cytosol, and Rac1 and PAK at the membrane. In the cytosol, we propose that $Rac1^{GDP}$:RhoGDI is activated, forming a $Rac1^{GTP}$:RhoGDI:PAK complex, in which PAK does not appear to be active (Figure 1d). We cannot formally exclude the possibility that PAK is complexed only with Rac1 or with other proteins; note that available PAK antibodies identified MDCK PAK by immunoblotting but not by immunoprecipitation. In sup-

port of the Rac1:RhoGDI:PAK complex, we note that PAK binds only active Rac1 [9], while RhoGDI binds both active and inactive Rac1 [8]. Furthermore, RhoGDI and PAK both bind Rac1 via multiple domains [19, 20], allowing for the formation of a trimeric complex. In the absence of signals to translocate the Rac1:PAK complex to the membrane, GTP hydrolysis would result in dissociation of the trimeric complex into $Rac1^{GDP}$:RhoGDI and PAK. Serum could activate factors at the membranes that are necessary to break the interaction between Rac1 and RhoGDI, allowing the $Rac1^{GTP}$:PAK complex to translocate to specific membrane sites. Of many structures that stained with PAK antibodies, only staining at ruffling membranes was lost following serum withdrawal (Figure 2f), which coincided with the loss of the Rac1:PAK complex from membranes (Figure 2d). We conclude that the Rac1:PAK complex localizes to ruffling membranes in response to serum. Dissociation of the Rac1:PAK complex would leave monomeric Rac1 to bind RhoGDI, displacing Rac1 into the cytosol [8] and leaving PAK to further act on downstream targets.

These results show that serum activation of Rac1 induces the localized membrane recruitment of a distinct effector complex with a specialized function. This suggests that, as a general mechanism, the mechanism of Rho GTPase activation selects defined interactions in order to elicit a particular biological function.

Materials and methods

Cell fractionation

MDCK cells [14, 17] were washed with ice cold Ringer's buffer supplemented with 1.8 mM $CaCl_2$, scraped off the petri dish in this buffer, and then disrupted by 15 passages through a ball bearing homogenizer in buffer A (120 mM KCl, 20 mM HEPES [pH 7.4], 2 mM $MgCl_2$, 0.25 M sucrose, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 10 μg/ml antipain). The homogenate was cleared by centrifugation at 2,000 × g for 5 min at 4°C. The resulting postnuclear supernatant was separated into cytosol and membranes by differential centrifugation at 100,000 × g for 45 min at 4°C. The pellet fraction was extracted on ice for 1 hr in buffer A containing either 1.0% (w/v) octyl-β-D-glucopyranoside, 1 M KCl, 4 M urea, 1% (v/v) Triton X-100, or bicarbonate buffer (pH 11.5).

Gradient centrifugation

Samples in buffer A were diluted 1:1 with 60% (v/v) iodixanol (OptiPrep™, Nycomed) and separated by isopycnic centrifugation in a VTi80 rotor (Beckman) at 67,500 rpm for 1 hr at 4°C; gradients were fractionated into ten 600 μl fractions. For rate zonal centrifugation, samples were separated into 4 ml continuous sucrose gradients comprising 7.5%–25% or 10%–40% (w/v) sucrose in the SW60 rotor (Beckman) at 48,000 rpm for 9 hr at 4°C; gradients were fractionated into twenty 200 μl fractions.

Immunoprecipitation

Rate zonal gradient fractions were incubated for 1 hr at 4°C with 1 μl of phosphoserine antibody (Sigma, PSR-45) and then for 1 hr on a rotator at 4°C with protein G sepharose. Sepharose beads were washed three times with 1 ml volumes of buffer A, and then boiled in 2× SDS-PAGE buffer for 10 min.

Western blotting

Proteins were separated by SDS-PAGE (15% for Rac1 and RhoGDI, or 7.5% for PAK) or native PAGE (5% or 7%) and processed for Western blotting. Antibodies used for Western blotting were: αRac1 (Transduction Labs), 9E10 (αmyc epitope tag), αRhoGDI (Transduction Labs), and PAK (C-19, Santa Cruz). C-19 recognizes α, β, and γ PAK isoforms; in MDCK cells, C-19 primarily recognized a 62-kDa band, indicating that γPAK is the predominant PAK isoform in these cells. Antibody/protein binding was detected using horseradish peroxidase–conjugated secondary antibodies followed by ECL.

Immunofluorescence

Cells were fixed on ice for 15 min with 4% (w/v) paraformaldehyde, permeabilized with 0.075% (w/v) saponin, and incubated with rhodamine-phalloidin and PAK antibodies (C-19) followed by FITC-conjugated goat anti-rabbit secondary antibodies (Jackson Labs).

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